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# Application of residual sludges from wastewater treatment technologies for construction of biofertiliser

Mihaela Belouhova[1,2], Dobromira Yaneva[1,2], Yana Topalova[1,2]

**1** *Department of General and Applied Hydrobiology, Faculty of Biology, Sofia University St. Kliment Ohridski, 8 Dragan Tzankov Blvd, Sofia 1164, Bulgaria* **2** *Center of Competence "Clean technologies for sustainable environment – waters, wastes, energy for circular economy", Sofia University St. Kliment Ohridski, 15 Tzar Osvoboditel Blvd., Sofia 1504, Bulgaria*

**Corresponding author**: Mihaela Belouhova (mihaela.kirilova@uni-sofia.bg)

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## Abstract

To stimulate plant development in phytoremediation or in the cultivation of non-food crops in potentially contaminated soils, a biotechnologically created product could be applied. The aim of this study was to explore the possibility of creation of biofertiliser, based on activated sludge combined with bacterial strain with detoxifying and plant growth promoting properties. The presented study is focused on the effect of phenol in the following concentrations: 5 mg/l, 100 mg/l, 250 mg/l, 500 mg/l and 1000 mg/l on the metabolic activity of *Brevibacillus laterosporus BT271*. The gradual increased concentration of phenol was used to study the metabolic activity of mineralised activated sludge and *B. laterosporus BT271*. The CTC/DAPI staining showed high activity of the bacteria even at the highest concentration. The greatest amount of biomass was accumulated at 5 mg/l phenol ($4.44 \times 10^7$ cells/ml). At this toxicant concentration, a total dehydrogenase activity of $5.72 \times 10^{-4}$ µg H+/ml*min was found. Studies of the metabolic activity of microorganisms in experiments involving a combination of mineralised activated sludge, *B. laterosporus BT271* and phenol at three concentrations (5 mg/l, 250 mg/l and 1000 mg/l) showed the highest value for dehydrogenase activity in the variant with average phenolic concentration (up to $6.39 \times 10^{-6}$ µg H+/ml*min. The results proved the detoxification potential of *B. laterosporus BT271* when different concentrations of phenol were present. The combination of a mineralised activated sludge and selected highly active biodegrading *B. laterosporus BT271* showed valuable properties of detoxification and metabolic activity and keep these potentials up to 1000mg/l phenol.

## Keywords

Bioremediation, *Brevibacillus laterosporus BT271*, mineralised activated sludge, phenol

## Introduction

Soils are a key resource for the existence and maintenance of normal human life and the planet. They are, therefore, the focus of the EU's Green Deal, adopted in 2019. According to this document and the European Commission's 'Towards Zero Pollution for Air, Water and Soil' action plan, Europe must have achieved zero soil pollution by 2050 (European Commission 2019, 2021). At the same time, in addition to the absence of pollution in the maintenance of soil fertility, the principles of the circular bio-economy need to be introduced (European Commission 2019). These ambitious goals will be difficult to achieve in the current economic and health crisis, combined with the lack of technology readiness in the various industries. Therefore, it is necessary to look for innovative solutions that allow environmentally-friendly treatment of soil resources and subsequent maintenance of fertility in them. One of the important directions in this subject is the creation of a less toxic environment. When it comes to soils, this involves the elimination of toxic substances, such as pesticides. Another aspect is the use of treated water in irrigation in agriculture. This again carries risks for the entry of toxic substances, antibiotics, steroid hormones and other xenobiotics into the soil. They must be degraded by the soil microflora or by the addition of biofertilisers. This requires a special focus on innovation activities related to the targeted management of detoxification processes in soils used for intensive or organic farming.

Activated sludge is formed during the treatment of domestic wastewater and, after stabilisation and decontamination, can be used for soil treatment (Gray 2010; Dimkov et al. 2017). They are alternatives to conventional fertilisers and offer a circular solution for solid waste from water treatment – stabilised sludge (Meuser 2013). These innovative and complex biofertilisers can be used for the reclamation of soils that are contaminated or damaged by human activity (Banov et al. 2020). The activated sludge is rich in both humic substances and micro-, macronutrients. It also contains microorganisms that are beneficial for the soil.

However, the bacteria in the activated sludge for recovery have low detoxification activity and are adapted mainly to the biodegradation of only trivial contaminants. In the case of soil contamination with organic pollutants, it is necessary to enhance the target detoxification activity by adding highly active biodegradants, which perform the biodegradation of toxic contaminants at a high rate and with a minimum degree of inhibition. This approach to bioaugmentation is used for contamination with petroleum products, pesticides, insecticides, pharmaceuticals, PFAS, antibiotics and other products, xenobiotic soil contaminants and residues in recycled water for irrigation (Hong et al. 2020; Li et al. 2021). In these bioaugmentation procedures for the detoxification activity of biological fertilisers, consortia (Varjani and Upasani 2019; Laothamteep et al. 2022) or individual bacterial cultures belonging to various genera can be used - *Pseudomonas* (Ramadass et al. 2018), *Enterobacter* (Koolivand et al. 2020), *Micrococcus* (Nwankwegu and Onwosi 2017), *Burkholderia* (Morya et al. 2020), *Bacillus* (Banerjee et al. 2019), *Shewanella* (Zou et al. 2019), *Xanthomonas* (Xu et al. 2018) or *Alcaligenes* (Haouas et al. 2021). Of particular interest are the bacteria of the genus *Brevibacillus*

(Arya and K. Sharma 2015). They have pronounced bioremediation properties against various organic and inorganic pollutants (Wei et al. 2019; Jebril, Boden and Braungardt 2021). These microorganisms are also of particular interest due to the possibility of their bioremediation detoxification properties to be combined in microbial preparations with the effect of protection against phytopathogens and enhancing plant growth (Ahmed et al. 2018). This study aims to explore the possibility of creating a biofertiliser, based on activated sludge combined with a bacterial culture from g. *Brevibacillus* with detoxifying and possible plant growth-promoting properties.

## Materials and methods

### Experimental design

The present study is focused on the exploration of the potential of a bacterial culture (*Brevibacillus laterosporus BT271*) for the creation of a biofertiliser with biodetoxifying properties. A combination of the highly-active bacteria with activated sludge that is conventionally used for the restoration of soil, will give added value to the future bioproduct in the trend of the circular solutions. A waste (the residual activated sludge) will be transformed into a valuable biotechnological product. It can be applied for the restoration of the damaged environment. The here-described experiments include the first stages of development of such a product. The *B. laterosporus BT271* metabolic activity in the presence of a model xenobiotic (phenol) was evaluated. The volume of the used 14$^{th}$ hour bacterial culture in the experiments at this stage was 4%. Nutrient broth was used. The microorganisms were cultured at 30 °C and aerated. Phenol was added once at zero hour in the following five concentrations: 5 mg/l, 100 mg/l, 250 mg/l, 500 mg/l and 1000 mg/l. Critical control points (CCPs) were set at 0 hour and 6th hour. The metabolic activity of the combined residual activated sludge (rAS) and *B. laterosporus BT271* was studied. Activated sludge weighing 0.5 g was used. Highly active biodegradant was added as fresh biomass, which is 10% of the weight of the activated sludge. The microorganism was cultured at 30 °C and aerated. Phenol was added once at zero hour in the following three concentrations: 5 mg/l, 250 mg/l and 1000 mg/l. CCPs were also set at 0 hour and 6th hour. The metabolic activity of the combined residual activated sludge (rAS) and *B. laterosporus BT271* was studied. The bacterial activity and the activity of the combination with rAS was estimated on the accumulation of the biomass, the total dehydrogenase activity of the microorganisms and the intoxication effects, determined with fluorescence and digital image analysis.

*Brevibacillus laterosporus BT271* – the bacterial culture was isolated from contaminated soil close to an oil refinery (Lukoil Neftochim Burgas, Bulgaria) by Prof. Yana Topalova (Topalova 2009). This bacterial culture was adapted to the biodegradation of aryl-containing xenobiotics (phenol, nitrophenols, pentachlorophenol in high concentration). The culture possesses a broad spectrum of biodegradation activity towards 30 different xenobiotics. The culture can survive in high percentage after lyophilisation and quickly recovered detoxification activity after re-hydration. The bacteria in our experi-

ments were kept as a lyophilised preparation prior to the experiments. They were cultivated in Nutrient agar (HiMedia, India). For the experiments for the intoxication effects, 18 h bacterial culture was used.

### Activated sludge

The used activated sludge was residual activated sludge (rAS), treated with CaO (quicklime) and ready for utilisation in agriculture. The rAS was taken from the WWTP on Sofia City, Bulgaria.

### Phenol

The model toxicant used in the study was phenol since its derivatives are major environmental pollutants. It was supplied by Fluka Analytical (Switzerland). Five phenol concentrations in the range 5–1000 mg/l were used in the experiments.

### Methods

The accumulation of the biomass was monitored by the optical density at 430 nm. The total dehydrogenase activity was determined by the method of Lenhard (Lenhard et al. 1964).

CTC/DAPI-based analysis was applied for determination of the bacterial metabolic activity and detection of changes in the morphology of the cells. It is based on the use of two fluorescent dyes. CTC or 5-cyano-2,3-ditolyl tetrazolium chloride is a tetrazolium salt that has no fluorescent properties. In the living cells, it is reduced to CTC-formazan that emits a red fluorescence signal. In these experiments, it was used in 5 mM concentration. DAPI (4',6-diamidino-2-phenylindole) is a fluorescent dye that binds DNA and emits a blue fluorescent signal. In concentration 1 μg/ml, it can be used as a staining method for all the biomass in the samples. By using CTC and DAPI simultaneously, information about the live cells in the whole sample was obtained. Fluorescence images were taken by a "Leica" DM6 B microscope. They were further processed with the software DAIME (Daims et al. 2006). The share of the live cells, their intensity and size (or the area of the cells) were calculated with the programme by using custom threshold segmentation. All the analyses were performed in three independent repetitions.

## Results

In the experiments performed, the accumulation of biomass in *B. laterosporus BT271* under optimal conditions for development was studied. The Nutrient medium provided a sufficient concentration of easily-degradable substrates and a lack of toxicants. The obtained results are illustrated in Fig. 1. The bacterial culture reaches its maximum number of cells at the 20$^{th}$ hour of its growth (3.25 × 107 cells/ml), after which it passes into a stationary phase of growth and a death phase. These data were also supported by the activity of dehydrogenase in the cells - the total dehydrogenase activity is highest

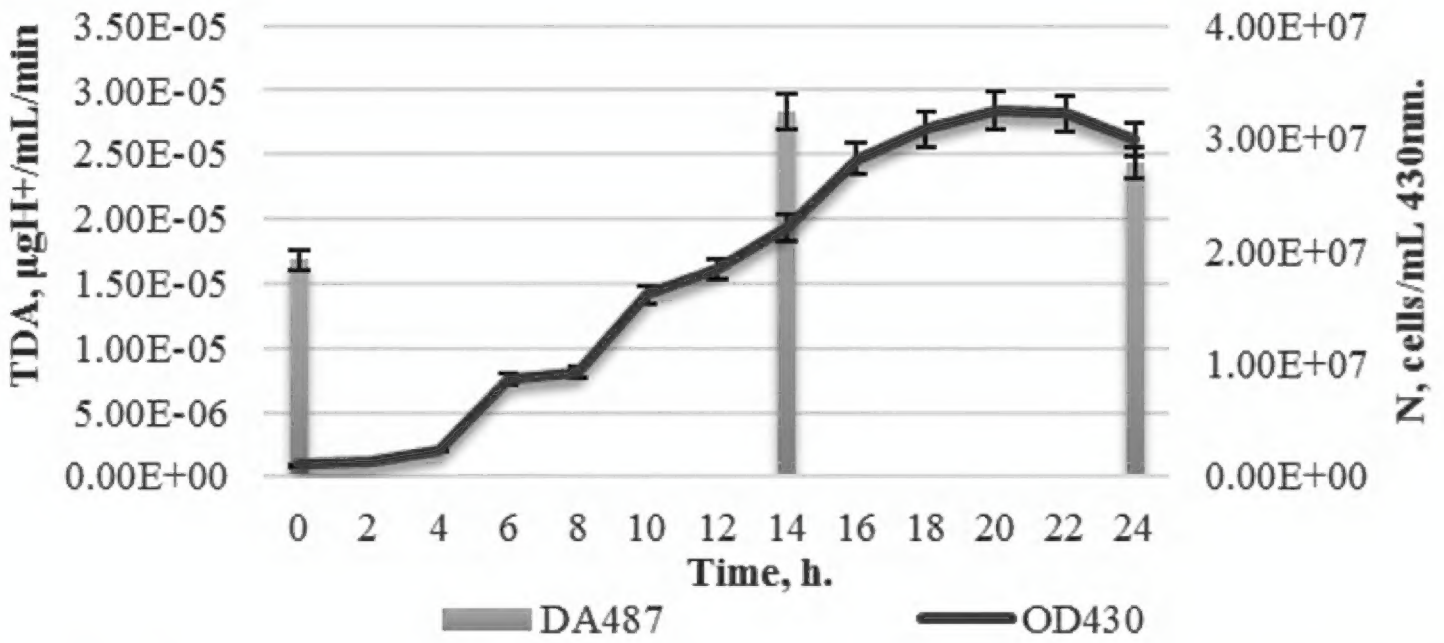


**Figure 1.** Biomass accumulation and total dehydrogenase activity (TDA) in *B. laterosporus BT271* (DA487 – dehydrogenase activity, measured at 487 nm; OD430 – optical density at 430 nm).

at the 14th hour (exponential growth phase). The value of this indicator exceeds TDA at the beginning of cultivation by 69% and by 16% at the 24th hour of the culturing.

In the further experiments for studying the detoxification properties of *B. laterosporus BT271*, cultures in the late exponential phase (18 hours) were used. They had the maximum number of bacterial cells with the most active enzymes.

In Fig. 2 A, the accumulation of biomass in *B. laterosporus BT271* in the presence of five phenol concentrations is presented. The presented data show that the bacterial culture degrades phenol in wide range of concentrations - from 5 mg/l to 1000 mg/l. The number of cells in the presence of phenol reached $4.44 \times 10^7$ cells/ml in the presence of 5 mg/l phenol. The largest increase occurred when the microbial count was recorded at low concentrations (16% for 5 mg/l and 13% for 100 mg/l). This is due to the lower inhibitory effect of the phenol in these concentrations. In contrast to the phenol experiments, in the cultivation of the bacterial culture in the absence of the phenol (control), a decrease of 3.25% was registered between the number of cells at 18 hours and 24 hours (Fig. 1).

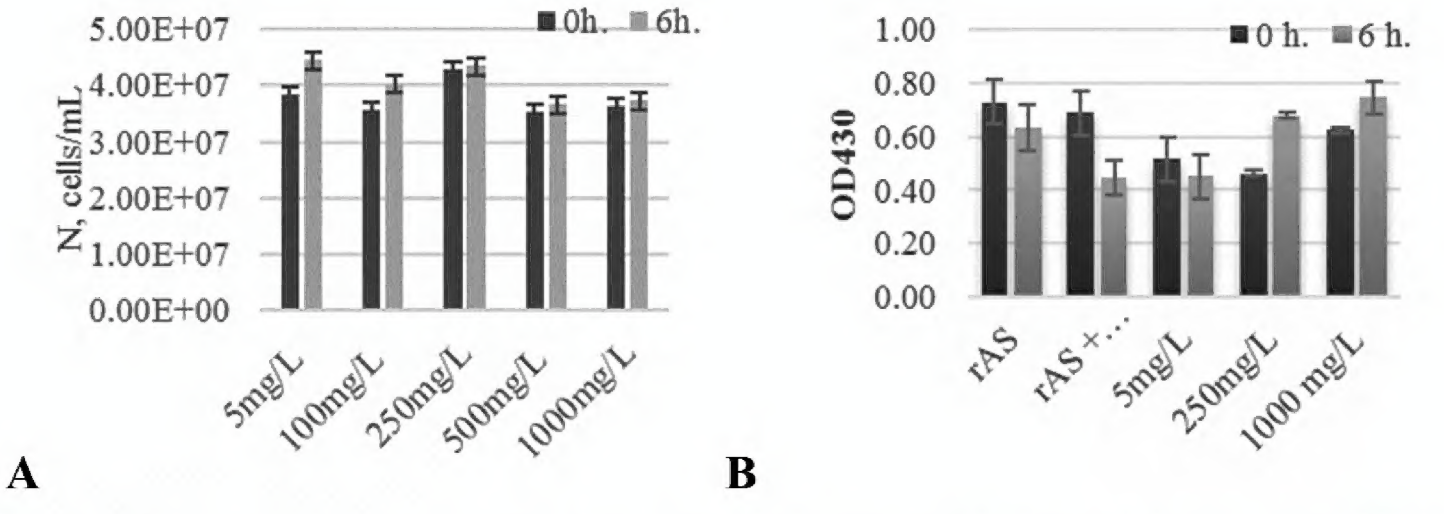


**Figure 2.** Change of the biomass accumulation in: **A** *B. laterosporus BT271* and **B** *B. laterosporus BT271* and rAS in presence of phenol in concentrations 5–1000 mg/l.

At the highest phenol concentrations, an increase in accumulated biomass was also found, although significantly less - 3.4% for 500 mg/l and 2.5% for 1000 mg/l phenol.

In addition to determining the biomass accumulated from *B. laterosporus BT271* in the presence of phenol, the change in absorbance when combining the bacterial culture with activated sludge for recovery was also investigated. This information can be used as an indirect indicator for the increase in biomass of the target detoxification culture. The obtained data are illustrated in Fig. 2B. At the high concentrations of the phenol, a significant increase was registered (average 34%). It is greatest at the addition of 250 mg/l phenol, but remains significant even at 1000 mg/l phenol.

The metabolic activity of the bacteria in the presence of phenol was determined, based on the total dehydrogenase activity. The results obtained showed that, in *B. laterosporus BT271*, there is a significant activation of metabolic processes in the presence of phenol (Fig. 3A). At the beginning of the experiment, the measured TDA was from $1.06 \times 10^{-5}$ μg $H^+$/ml*min to $1.06 \times 10^{-4}$ μg $H^+$/ml*min. After 6 hours of incubation, the values of this indicator increased from $7.58 \times 10^{-5}$ μg $H^+$/ml*min to $5.74 \times 10^{-4}$ μg $H^+$/ml*min. The highest increase was registered when 5 mg/l phenol was present - 7.3 times. It was lowest when 1000 mg/l phenol was applied - 2.7 times.

Fig. 3B represents the data on TDA for the combination of the highly active microorganisms *B. laterosporus BT271* with activated sludge for recovery. At the beginning of the experiment (0 h.), the values of this indicator were very low - they were at the lower limit of detection for this method. The only exception was the higher activity recorded in bacteria with the addition of 250 mg/l phenol. This is most likely due to the rapid activation of bacterial metabolism in the presence of this intermediate concentration of the toxicant.

After incubation for 6 hours, an increase in the activity of microorganisms was registered in all tested variants. This is from 30% (at 250 mg/l phenol) to 32 times (at the highest concentration of the toxicant). The effect recorded in the activated sludge controls was most likely due to the rehydration of the cells in the activated sludge and their incubation at the optimum temperature (30 °C).

The analysis of the metabolic activity, based on CTC, showed that, at most of the used concentrations, a slight decrease in the indicator was registered (on average by 14%)

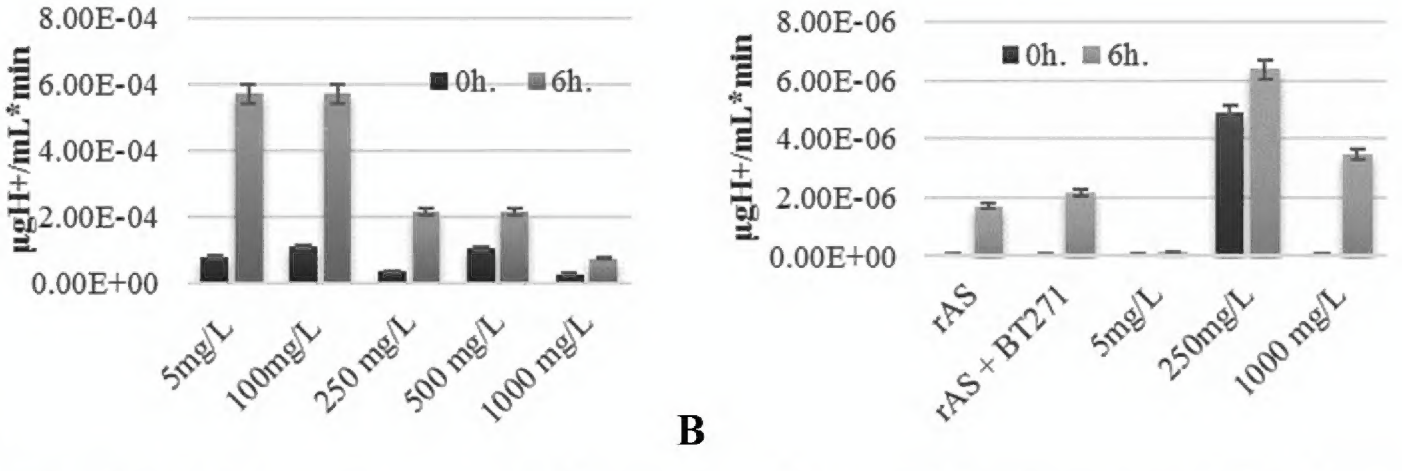


**Figure 3.** Total dehydrogenase activity in **A** *B. laterosporus BT271* and **B** *B. laterosporus BT271* and rAS in presence of phenol in concentrations 5–1000 mg/l.

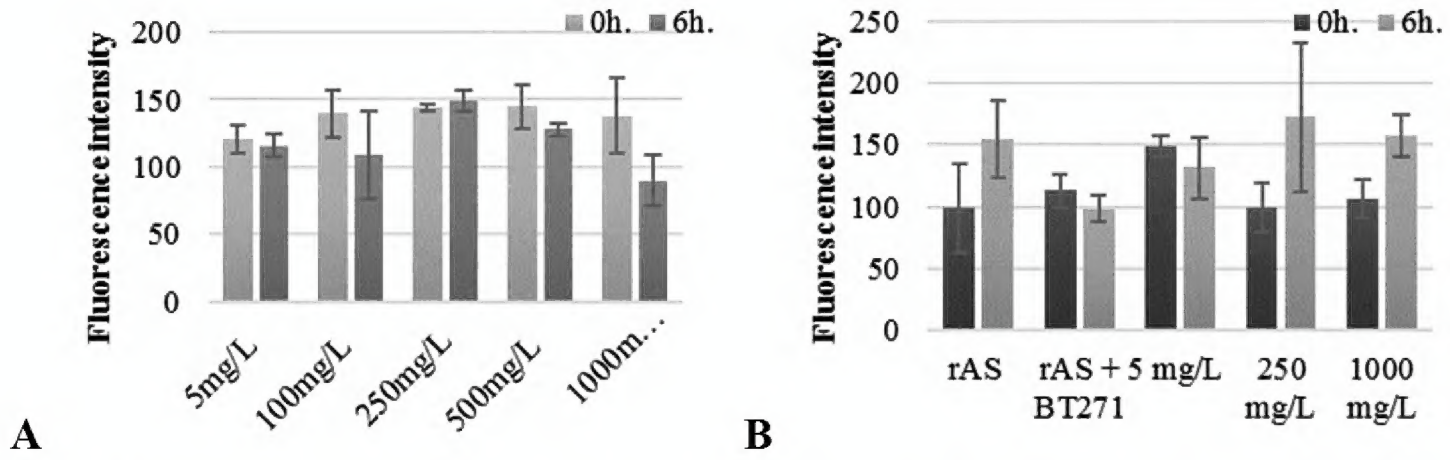


**Figure 4.** Fluorescence intensity in **A** *B. laterosporus BT271* and **B** *B. laterosporus BT271* and rAS after 6-hour incubation in presence of phenol.

(Fig. 5). The difference in the fluorescence intensity of CTC varies from 3% (at 5 mg/l phenol) to 35% (at 1000 mg/l). An increase in fluorescence intensity (by 4%) was found only at a phenol concentration of 250 mg/l. The differences in the results obtained with the TTC-based method are most likely related to the differences in methodology. The CTC method provides information directly about the individual cells in the samples. The information from the TTC method represents the activity of all bacteria in the sample. It is based on the extracted formazan, the concentration of which is determined spectrophotometrically. Additionally, in the TTC-TDA analysis, an easily degradable substrate is added (glucose). This activates the metabolism of the cells. The data from this analysis can be considered as information about the metabolic activity of certain microorganisms at the optimal concentration of the dehydrogenase substrate. On the other hand, the CTC method shows the metabolic activity of the cells directly under the conditions of the experiment, i.e. not to what extent their metabolic functions are preserved (as in the TTC-TDA method), but to what extent they are active in the conditions of intoxication.

Thus, it is clear that, in the presence of phenol in different concentrations, the bacterial culture *B. laterosporus BT271* had high metabolic activity, estimated as TDA, based on the reduction of TTC. However, under the conditions of the experiment, the activity of the culture decreased slightly after the 6-hour incubation with phenol. An exception was the concentration of 250 mg/l, at which higher fluorescence intensity was detected (Fig. 4A). In addition, it showed an increase in the average cell size (by 44%). At the same time, at the highest concentration used (1000 mg/l), there was a decrease in intensity by 34% and a decrease in cell area by 86% - an indication of intoxication changes in bacterial cells at this concentration.

In Fig. 6, fluorescent pictures of CTC staining of the residual activated sludge before and after the inclusion of *B. laterosporus BT271* bacteria are shown. The images represent the increased number of living cells after the addition of bacteria. The data obtained were confirmed by the fluorescence intensity after 6 hours of incubation. It is 73% higher in the presence of 250 mg/l phenol. The highest concentration of the toxicant increased the metabolic activity of the microorganisms by 48% (Fig. 4B). These results were also confirmed by the TTC method for the determination of TDA.

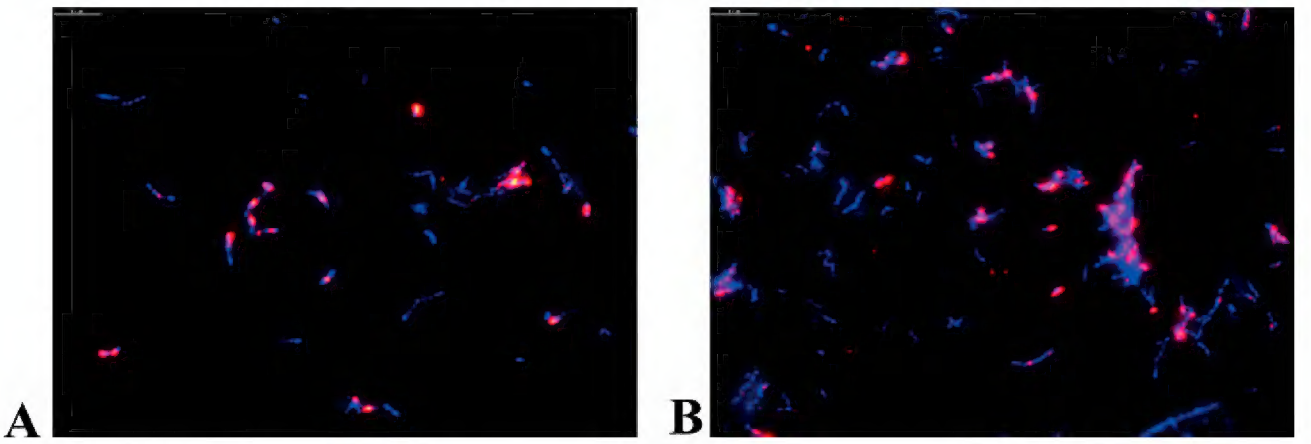


**Figure 5.** CTC/DAPI staining of *B. laterosporus BT271* in the presence of 5 mg/l phenol: **A** 0 h; **B** after 6 hours of incubation.

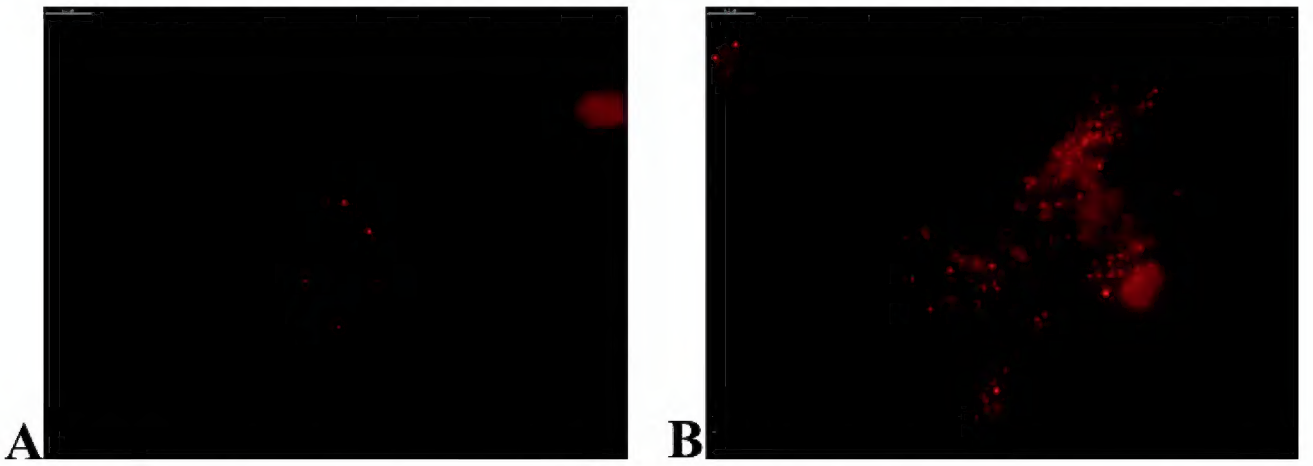


**Figure 6.** CTC staining demonstrating the bacterial abundance and metabolic activity in: **A** residual activated sludge; **B** residual activated sludge with added *B. laterosporus BT271*.

In both methods used to study the metabolic activity of microorganisms in the combination of activated sludge and *B. laterosporus BT271*, the lowest effect was recorded in the presence of 5 mg/l phenol.

The results of the CTC analysis also show an increase in the metabolic activity of the sludge after rehydration (the control with activated sludge only). In this experimental result, an increase in metabolic activity by 36% was found. The data obtained showed that, when *B. laterosporus BT271* was added to this sludge at the highest concentrations used (250 mg/l and 1000 mg/l), a significantly higher increase was achieved compared to the rehydrated sludge alone (61% on average). This is indicative of the high biodetoxification potential of the combination of activated sludge and *B. laterosporus BT271*.

## Discussion

The data discussed in the Results section showed that the combination of activated sludge and bacterial culture of *Brevibacillus laterosporus BT271* has a high potential for use in biodetoxification procedures. These are required for environmental pollution with various pollutants. Soils have been identified by the EU as a resource that must be protected and purified because of their critical importance for "human health, the state of the economy and the production of food and new medicines" (European

Commission 2020). One of the most common techniques for soil decontamination (bioremediation) is bioaugmentation - the addition of highly active biodegradants to eliminate the respective contamination. Numerous bacterial genera have been identified as suitable for use in bioremediation procedures – *Bacillus* (Hsieh et al. 2020), *Pseudomonas* (Alhefeiti et al. 2021), *Shewanella* (Zou et al. 2019), *Xanthomonas* (Xu et al. 2018), *Alcaligenes* and *Brevibacillus* (Arya and K. Sharma 2015) and others.

The high potential for application of *Brevibacillus* bacteria was also confirmed in the experiments of the present study. *B. laterosporus BT271* increased in number despite the inhibitory effect of phenol applied in five increasing concentrations (5–1000 mg/l) (Fig. 2). At the lowest concentrations of the toxicant, its inhibitory effect was the weakest and the increase in the quantity of the detoxification culture was up to 16%. At the highest concentration (1000 mg/l), it was 3%. At the same time, during the cultivation on the Nutrient medium, a decrease of the biomass was registered at the 24$^{th}$ hour. The data obtained demonstrate that the culture can use the toxic phenol as a carbon source at all applied concentrations, which increases the number of cells, respectively the accumulation of the biomass of the detoxifying bacterial culture. Other authors also have established the applicability of *Brevibacillus* in the biodegradation and bioremediation of phenol and phenolic derivatives (Abu Talha et al. 2018; Wei et al. 2019).

In addition to specially-selected bacterial cultures, in practice, activated sludge from wastewater treatment plants is traditionally used as a biofertiliser. It is formed in the course of water purification after which it is subjected to stabilisation and decontamination and can be applied directly to soil enrichment in agricultural and bioremediation procedures (Bitton et al. 2016). Activated sludge is traditionally used for fertilisation because it is a natural source of nutrients (Abdollahinejad et al. 2020). When these sludges are enriched with an appropriate amount of biomass from microbial cultures with detoxifying properties, biofertiliser with combined valuable properties is obtained - for enrichment of the soil and soil fertility and simultaneous disposal of residual toxic contaminants in the soil.

The targeted combination of mineralised sludge and the detoxification culture of *B. laterosporus BT271* in the present study resulted in a very good result in terms of activity of the metabolic processes. As commented in the "Results", the combination with activated sludge and *B. laterosporus BT271* led to an increase in biomass accumulation by up to 47%. The low effect found in the controls and at 5 mg/l phenol is most likely due to the lack of sufficiently easily-degradable nutrient sources.

The presence of phenol in different concentrations has an effect, not only on the accumulation of biomass, but also on the activity of bacteria, both only on *B. laterosporus BT271* and in the combination of these microorganisms with the activated sludge. The rate of metabolic transformations increased from 2.7 to 7.3 times in the presence of phenol. When combining the highly-active microorganisms with activated sludge and the effect of phenol in a concentration of 1000 mg/l, it causes an increase in metabolic activity by 32 times. This result, as well as the rapid activation of metabolic processes and the high value of TDA at 250 mg/l phenol, are an indication of the prospects for the combination of activated sludge and *B. laterosporus BT271* for the creation of biofertiliser with valuable combined properties for soil detoxification and increase in soil fertility.

Fluorescence analysis showed that, although the individual *B. laterosporus BT271* cells decreased their activity after the addition of phenol, in the presence of activated sludge the total activity of the system increased by up to 73%. Thus, the use of preparation with *B. laterosporus BT271* and activated sludge in bioremediation activities could achieve a high rate of elimination of toxicants and, at the same time, would influence the soil fertility in a favourable direction in a way close to nature according to the rules of the circular economy and the principle "nature knows best".

## Conclusion

The results, presented so far, were focused on the study of the prospects for the creation of biofertiliser with combined activated sludge and specially-selected microorganisms with detoxifying activity (*B. laterosporus BT271*). They showed that the accumulation of biomass and bacterial metabolic activity not only remain high, but also increased by 32 times in the presence of phenol in high concentrations (up to 1 g/l). This suggests that the application of the preparation with AS and *B. laterosporus BT271* will have accelerated biodegradation in areas contaminated with xenobiotics with a cyclic structure (phenol and phenolic derivatives). It will be possible to use the synergistic effect of the introduction of nutrients (in the form of activated sludge) and specially-selected biodegradants of xenobiotics (*B. laterosporus BT271*), which also have protective properties against plants. Further experiments will be continued in the direction of testing the preparation in conditions close to the real ones.

## Acknowledgements

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